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# PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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*Additional inventors are being named on the* one *separately numbered sheets attached hereto*

**TITLE OF THE INVENTION (500 characters max)**

Bone Marrow Progenitor Cell Recruitment to an Ectopic Mesh Implant Using Controlled Release of Cytokines and Growth Factor

*Direct all correspondence to:* **CORRESPONDENCE ADDRESS**

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**ENCLOSED APPLICATION PARTS *(check all that apply)***

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☐ Drawing(s) *Number of Sheets* ☐ Other (specify)

☐ Application Data Sheet. See 37 CFR 1.76

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*Respectfully submitted,*

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Date January 28, 2004

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# Bone Marrow Prog nitor C ll R cruitment to an Ectopic M sh Implant using Controll d R lease of Cytokines and Growth Factors

D.M. Ferris and E. Mathiowitz

## SUMMARY OF WORK

The possibility of augmenting the body's own now-recognized bone marrow reservoir of stem and progenitor cells through the controlled release of specific cytokines and growth factors is an intriguing therapeutic option that could significantly improve cardiovascular repair. The work outlined below demonstrates for the first time that drug delivery systems delivering intact, bioactive molecules in a localized, specific space can recruit progenitor cells from the bone marrow to an ectopic site or implant. Fourteen normal 7 week old B6;129S mice underwent sub-lethal total body irradiation and then received a bone marrow transplant, via the tail vein, comprised of $10x10^6$ bone marrow cells (pooled from four Rosa26 donor mice) containing the bacterial *lacz* gene. Replacement of normal host bone marrow by *lacz* marked bone marrow was confirmed after 8 weeks using flow cytometry analysis. Nine weeks post-bone marrow transplant, nylon mesh implants containing microspheres loaded with appropriate growth factors were implanted into the dorsal subcutaneous space. Controls included empty nylon mesh (n=2), mesh and BSA control spheres (n=3). Experimental groups included mesh + VEGF spheres (n=3), mesh + GM-CSF spheres (n=3) and mesh + VEGF + GM-CSF spheres (n=3). After 21 days, animals were sacrificed, and implants and adjacent tissue were removed and processed for histological analysis. Preliminary results of three cohorts (n=3 per group) revealed marked recruitment of *lacz*+ cells to implants containing both VEGF and GM-CSF loaded microspheres. Cell recruitment was specific to this implant and was not seen in any other group studied, including groups containing the individual cytokine or growth factor. Remarkably, recruited *lacz*+ cells were "captured" entirely inside the mesh implant and could easily be removed for further analysis or culture.

## POTENTIAL APPLICATIONS AND CLAIMS OF WORK

The range of possible applications of this technology is broad. In short, this system could be applied to any situation that is need of <u>augmentation in vascularization</u>:

- myocardial infarction
- peripheral vascular disease
- diabetic ulcers
- ischemic bowel disease
- transient ischemic attacks (e.g. stroke).

This technology could also be applied to aid the success of various <u>tissue engineering applications</u>:

- Artificial skin
- Small diameter vascular grafts
- Stents

- Any device/tissue/organ that would benefit from enhanced vascularization.

Patients undergoing treatments for cancer could have such cells collected prior to radiation or systemic chemotherapy with the idea that these cells could be "saved" and administered back to the patient to augment the healing process later. This recruitment process could be used to recruit cells of the immune system to a "permissive microenvironment" whereby the body's own immune system could be stimulated and enhanced to fight either cancer and deadly viruses like HIV.

If this technology is indeed recruiting pluripotent stem cells from the bone marrow of an adult animal to a specific location, even more applications become possible. For example, this mesh bag could be easily removed and the cells cultured *ex vivo*. A source of pluripotent stem cells obtained through noninvasive means would be an incredible scientific feat. Such cells could potentially be applied to aid healing in any portion of the body including the following systems:

- Cardiovascular
- Gastrointestinal
- Pulmonary
- Musculoskeletal
- Neurologic
- Endocrine
- Hematologic

## INTRODUCTION AND BACKGROUND

Our research group here at Brown University is part of an interdisciplinary department comprised of personnel with expertise ranging from membrane formation, surgery, tissue engineering, biology, physiology and drug delivery. Our laboratory specializes in the controlled delivery of various molecules ranging from small peptides, growth factors, and drugs to DNA. We have developed many polymeric-based microspheres designed to deliver a variety of therapeutic molecules [1] [2] [3] and have experience with the design and production of small diameter vascular grafts [4-6]. Due to recent advances in the literature discussed below and some exciting results reported in our preliminary data section, we have been inspired to focus on the potential of using controlled release protein and cytokine delivery technology to recruit progenitor cells from the bone marrow in adult animals. The application of the knowledge gained through the completion of this work is broad, ranging from innovative new avenues to cure ischemic tissue to the endothelialization of such biomaterial surfaces as vascular grafts and left ventricular assist devices (LVAD).

In the last 5-10 years there has been numerous data to suggest the presence of endothelial progenitor cells (EPCs) that can be recruited from the bone marrow in adult animals and in humans [7]. Three main approaches have thus far been documented and explored in the literature to acquire or recruit these cells in an adult animal, but each approach offers significant limitations if it is to be applied to humans in a successful

clinical setting. These cells can either be selected using fluorescence-activated cell sorting with markers specific to progenitor cells from the bone marrow itself, isolated using the same technique from the bloodstream after exposing the animal to systemic recruiting factors, or finally recruited to a peripheral site after sequential administration of exogenous cytokines. The first two methods could involve a specifically selected cellular transplant to a patient, which would require the co-administration of immunosuppressive agents since the cells are known to contain classical MHC I and MHC II molecules. The third approach would involve at least daily administration of the appropriate cytokine to achieve the desired therapeutic effect and has the disadvantage that these cytokines traditionally have very short half-lives in the bloodstream. This issue could be easily surmounted, however, by releasing these cytokines in a controlled, steady fashion from biodegradable microspheres. Our laboratory has demonstrated the efficacy of this approach in several recent publications [8] [9] and is confident that the delivery of therapeutic agents in this fashion could have significant physiological effects.

Several papers have strongly suggested the therapeutic potential of these cells. For example, Murohara, et al., and Kalka, et al., have demonstrated that infusion of selected populations of cells from the bone marrow in animals improves angiogenesis in ischemic limbs [10, 11]. Furthermore, Dacron grafts implanted in dogs were shown to be endothelialized exclusively by cells from transplanted bone marrow [12]. Two studies reported in humans have also been significant. LVADs removed after 6 months were shown to be colonized by CD34+ and VEGFR2+ endothelial and hematopoietic cells, both markers for early progenitor cells [13]. In addition, autologous transplantation of bone marrow cells improved patient peripheral vascular disease [14]. Thus, this cell population clearly exists in the bone marrow and can greatly improve significant pathophysiological cardiovascular conditions. However, to-date, all experiments have involved a cell transplant of some sort instead of focusing on a method to augment and mobilize this special cell population within the same patient. The most likely candidates to improve the body's natural healing process are either angiogenic and vasculogenic growth factors, and/or specific cytokines that are known to have chemotactic effects on the cells of the bone marrow.

Initial animal studies in this field have experimented with the vasculogenic potential of bone marrow by either delivering whole bone marrow transplants or selected cell populations via intravenous or intramuscular administration. [14-18] These bone marrow derived EPCs appear to be attracted to angiogenic foci in the peripheral vasculature [15, 19]. Recent work has demonstrated the ability to mobilize and recruit these cells using adenoviral vectors expressing angiogenic factors and recombinant proteins like VEGF, angiopoietin 1 and stromal derived factor-1 into the bloodstream [20, 21]. Work by Asahara *et al.* and others [11, 22, 23] has also examined the factors that govern the recruitment of endothelial progenitor cells in the adult.

Growth factors and cytokines that play an important role in the recruitment of bone-marrow-derived EPCs include both VEGF and GM-CSF. VEGF has been shown to increase the circulating concentration of EPCs [24], is chemotactic for circulating endothelial progenitor cells (CEPs) [20], and is known to regulate and promote angiogenesis

and vasculogenesis in the adult [25]. GM-CSF is released by bone marrow endothelial cells in response to VEGF in order to promote the growth of hematopoietic cells [26]. Exogenous administration of granulocyte monocyte colony stimulating factor (GM-CSF) was shown to mobilize endothelial progenitors and contribute to neovascularization [27]. Thus, we hypothesize that the controlled release of cytokines like GM-CSF and angiogenic factors like VEGF from biodegradable microspheres could be able to attract and stimulate progenitor cell populations in patient populations that fail conventional medical and surgical therapy. Furthermore, these microsphere delivery systems could be injected into regions of ischemia or could be incorporated into an implant like a vascular graft or stent.

## MATERIALS AND METHODS

### Lacz+ bone marrow transplant

The general strategy for the bone marrow transplantation arm of this study can be seen in figure 1 below.

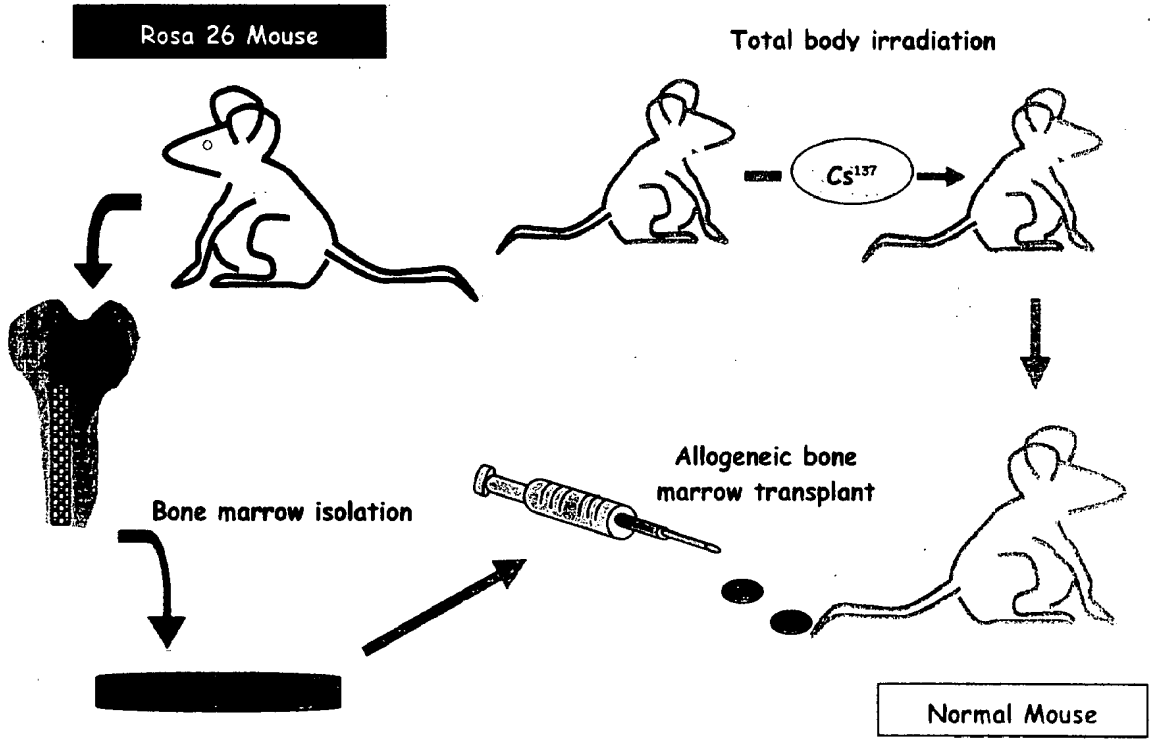


**Figure 1.** Bone Marrow Transplant. Bone marrow isolated from Rosa26 mice genetically modified to contain the bacterial lacz gene was collected and injected into age-matched, strain-matched "normal" mice via the tail vein. These normal mice underwent total body irradiation to remove endogenous bone marrow prior to the transplant.

Seven week old Rosa26 mice (B6;129S-Gtrosa26; The Jackson Laboratory, Bar Harbor, Maine, USA) that transgenically express the bacterial *lacZ* gene were used as donors for bone marrow transplantation. Seven week old B6;129S mice (The Jackson Laboratory)

were used as recipients for the bone marrow transplant via tail vein injection. These mice were given a sublethal radiation dose of 950 rads (2 doses of 425 rads, each spaced 3-4 hours apart) from a cesium137 source the day before bone marrow transplant. Mice received sulfatrim: 200 mg sulfamethoxazole and 40 mg trimethoprim (Alpharma, Baltimore, MD) one week prior to irradiation and for 4 weeks afterwards. Femurs and tibia from Rosa26 mice were removed under sterile conditions and the cells harvested and collected into sterile DPBS (Mediatech, Inc.). Approximately 10 x $10^6$ cells per mouse were transplanted into a total of 14 animals. Animals remained in sterile cage units post-transplantation until the time of surgery at 16 weeks of age.

Microencapsulation

**0.1% loaded VEGF and 0.1% BSA control microspheres**

Microspheres were fabricated with 50:50 poly (DL-lactide-co-glycolide, MW = 12,000) (Boehringer Ingleheim Inc. Germany) using a novel phase inversion technique developed in our laboratory. Briefly, a 50% solution of recombinant human vascular endothelial endothelial growth factor ($rhVEGF_{165}$), was combined with 10% bovine serum albumin and 10% Tween-20. This solution was added to a 0.001% polymer ethyl acetate solution and the two phase system vortexed and immediately shell-frozen, cooled in liquid $N_2$ followed by lyophilization for 48 hours. The dried polymer product was re-suspended in ethyl acetate (4% (w/v)) and the solution rapidly poured into petroleum ether (Fisher Scientific, Inc.) for formation of microspheres that were filtered and lyophilized for 48 hours for complete removal of solvent. Control spheres were made following exactly the same procedure with the exception of the replacement of VEGF with bovine serum albumin (Sigma Chemical, Missouri).

**0.2% loaded GM-CSF and BSA control microspheres**

poly-L-lactide (PLA, 8k) (Lactel, RL104.BI, batch # 33007) and PLA 24K (Lactel, 505-25-A) was used for the encapsulation of mGM-CSF (Biosource International). A similar encapsulation procedure was employed as above with the exception that methylene chloride was used in place of ethyl acetate. Control spheres were made following exactly the same procedure with the exception of the replacement of GM-CSF with bovine serum albumin (Sigma Chemical, Missouri).

Implant fabrication

0.8 cm x 0.8 cm squares of nylon mesh (SpecraMesh, CA) with a pore size of 20 microns were heat sealed on three sides and sterilized (Amsco Gravity 2051 autoclave). Appropriate microspheres were added to each "bag" and the fourth side heat-sealed prior to surgery. Groups included sham (empty nylon mesh bag only, n=2), mesh containing 6 mg of 0.1% and 2 mg of 0.2% BSA -loaded microspheres (n=3), mesh containing 6 mg of 0.1% VEGF-loaded microspheres (n=3), mesh containing 2 mg of 0.2% GM-CSF-loaded

microspheres (n=3) and a mesh containing both VEGF (6 mg) and GM-CSF (2 mg) microspheres (n=3).

**Animal Surgery**

Nylon pouches containing microspheres were implanted subcutaneously into the dorsal aspect of 16 week old B6129S mice (9 weeks post-bone marrow transplant). The mouse was anesthetized in an asphyxiation chamber with administration of inhalational isofluorane®. Anesthesia was maintained throughout the procedure by the administration of inhalational isofluorane® via a nose cone. A 1 cm incision was made just lateral to the spine in the right upper dorsal quadrant. After implant placement, the wound was closed using running sutures (Vicryl 6-0). After 21 days, animals were sacrificed using an overdose of metofane. Implants and adjacent skin were immediately removed, placed in OCT embedding medium (Sakura Finetek Inc. Torrance, CA) and quick-frozen on dry ice. A Leica CM1510 cryostat (Leica Microsystems, Germany) was used to take 14 micron frozen sections of implant cross-sections. X-gal staining to identify *lacz*+ cells was performed as follows.

Cryosections of the implant and adjacent skin were washed in PBS (pH 7.5) to remove OCT embedding medium and fixed for four minutes in 2% paraformaldehyde/0.5% glutaraldedhyde (Electron Microscopy Sciences, Fort Washington, PA). Slides were washed 3x in PBS (pH = 7.5), 2x in 2mM $MgCl_2$ PBS, 7.5 and 1x in staining solution (see below) without X-gal. Slides were then incubated at 37C for 8-16 hours with the X-gal staining solution prepared in PBS. The staining solution contained 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.3H_2O$, 2 mM $MgCl_2$, pH 7.5 and 1 mg/ml X-gal (Sigma Chemical Co., St. Louis, Missouri). Sections were then washed in PBS, dehydrated, and mounted in xylene:permount (50:50) (Fisher Scientific, Inc.) and viewed using a light microscope (Olympus, IX70) and pictures taken using a digital camera at magnifications from 1.25x - 20x.

## RESULTS

To confirm positive beta-galactoside staining, sections of mouse tissue from control mice (B6;129S) and Rosa26 were taken and cryo-sectioned as described above. No blue staining was seen in the negative control mouse tissue and skin from the Rosa 26 mouse contained positive blue staining denoting *lacz*+ cells in the dermis, blood vessels, plantar muscularis and in the hypodermis. (See Figure 2).

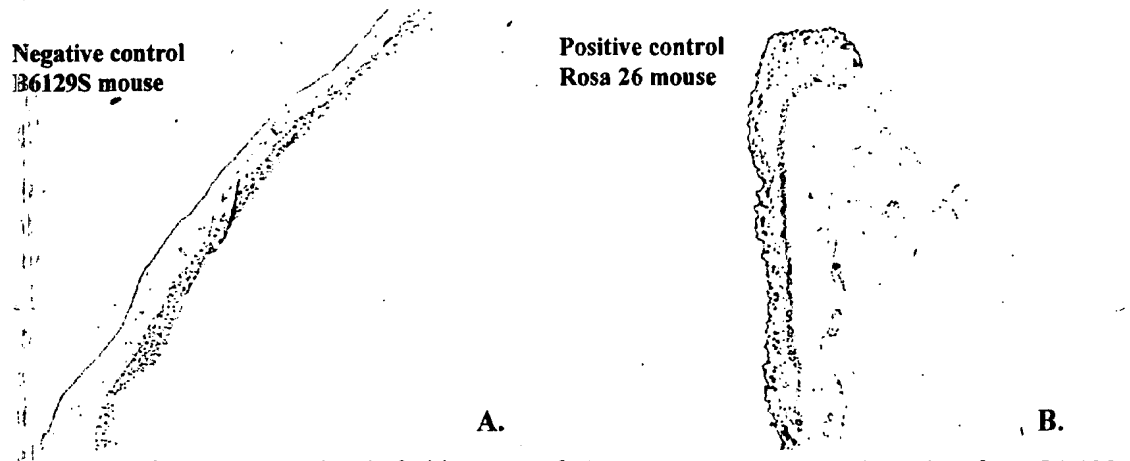


Figure 2. Light microscopy brightfield images of skin cross sections,. A. Skin taken from B6;129S mouse showed no positive Beta-galactosidase staining. B. Skin from Rosa26 mouse used for bone marrow transplantation showed positive staining throughout the tissue section. Magnification 1.25x

In order to rule out cell recruitment due to the nylon mesh itself or the degradation products of PLGA or PLA microspheres, two more controls were included in the study, a sham implant and a nylon mesh implant loaded with BSA control microspheres. Occasional blue staining was found along the cross-section of the sham implant, but no blue cells around found inside the implant. No evidence of *lacz* cells were found in skin adjacent to the BSA control implant or within the nylon mesh itself. (Figure 3).

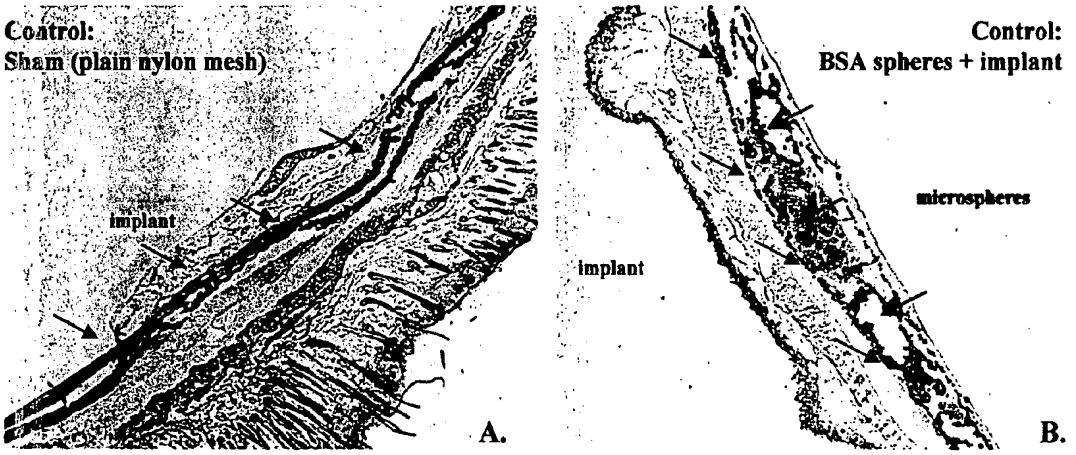


Figure 3. A. A few *lacz* cells can be found along the nylon mesh but no cells were found inside the implant. B. No *lacz* cells can be found inside the implant or in the skin adjacent to the implant. Magnification 1.25x.

To test the ability of GM-CSF and VEGF to individually recruit cells from the bone marrow to an ectopic site in an adult mouse, each factor was encapsulated into PLA or PLGA microspheres respectively, and implanted inside a nylon mesh bag for 3 weeks. No *lacz* cells positive cells could be found in sections of implants containing either growth factor (Figure 4.).

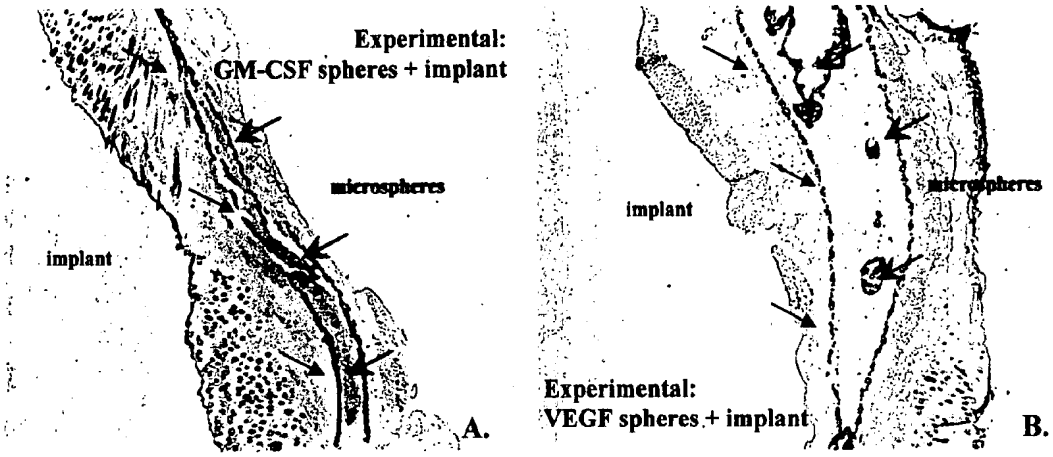


Figure 4. A. Implant containing PLA microspheres loaded with GM-CSF. Some cellular infiltration was found within the implant but no *lacz*$^+$ cells could be seen in adjacent tissue or within the implant. B. Implant containing PLGA microspheres loaded with VEGF. Significant cellular infiltration of the mesh implant can be found, but similar to the GM-CSF implant, no *lacz*$^+$ cells are present. Magnification 1.25x.

To test possible synergy between VEGF and GM-CSF, an implant containing both types of microspheres was tested as well. Cellular infiltration into the nylon mesh was significant and the majority of cells were *lacz*$^+$. No *lacz*$^+$ cells were found in the tissue adjacent to the implant (Figure 5).

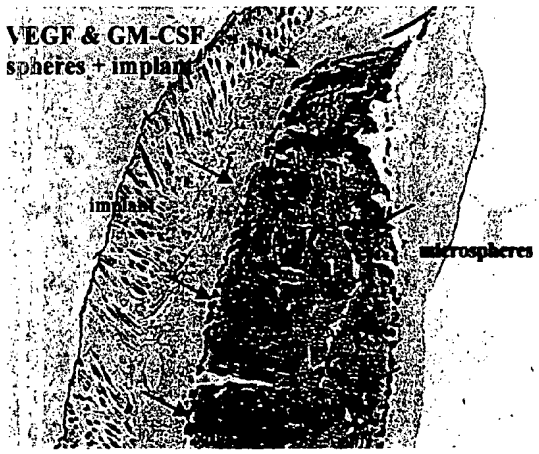


Figure 5. Cross-section of VEGF-GM-CSF nylon mesh subcutaneous implant. Black arrows denote implant edge and red arrows denote regions of microspheres. Significant cellular infiltration of the implant space was seen with the vast majority of cells *lacz*$^+$. No *lacz*$^+$ were found outside the margin of the implant. Magnification 1.25x.

## DISCUSSION/CONCLUSION

The work described above offers convincing evidence that the controlled release of growth factors and cytokines can not only mobilize cells from the bone marrow into the

bloodstream, but that these cells can then be recruited to a specific site remote from the vascular system in an adult animal. It is important to note that the mice at the time of surgery were 16 weeks old, an age considered quite advanced in mouse years. Furthermore, this study demonstrates that through specific study design, these cells can be entirely "captured" into a porous mesh bag and potentially extracted from the host animal. These cells could then be cultured *ex vivo* and transplanted back into the host or used therapeutically in another host. Further work must be done to characterize the exact nature of these cells, but the initial results are exciting and quite promising.

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